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## A sequential procedure for the quantification of biologically produced polyphosphate in sediment samples

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### ABSTRACT

#### A sequential procedure for the quantification of biologically produced polyphosphate in sediment samples

Polyphosphate (poly-P) is a phosphate storage compound widespread in prokaryotic and eukaryotic cells. Its quantification, however, is not yet well developed for sediment samples because of the difficulties associated with using a single extractant to extract a specific P compound without altering others. The present work uses an analytical procedure already developed to measure poly-P in algal cultures and later modified here for sediments to study poly-P in the sediment. The procedure uses the Ethylenediaminetetraacetic acid (EDTA) method for sequential P-fractionation. This approach ensures that the main inorganic phosphate compounds (i.e., Fe- and Ca-bound phosphate) are extracted prior to poly-P quantification. We applied this method to suspensions likely to contain biologically produced poly-P, such as laboratory cultures of *Synechocystis* PCC 6803 (Cyanobacteriaceae), *Anabaena variabilis* (Cyanobacteriaceae), and *Chlorella* sp. (Chlorophyceae) growing in a P-enriched medium, and activated sludge. According to this procedure, the concentrations of poly-P in *Synechocystis* PCC 6803, *Anabaena variabilis* and *Chlorella* cultures were 15.4, 6.2 and 7.2 % of the sum of all P-fractions, respectively, whereas activated sludge showed a lower percentage (3.7 %). Known volumes of these suspensions and a commercially available synthetic poly-P standard (Trimetaphosphate) were added to natural sediment. The P-composition of each sample was then compared in duplicate to the corresponding control samples (sediment alone). Poly-P in all control sediment samples was extremely low (0-2  $\mu\text{g g}^{-1}$  d.w.). It increased in all sediment suspensions to which biological samples had been added. The increase was particularly noteworthy for *Synechocystis* (up to 220  $\mu\text{g g}^{-1}$  d.w. of poly-P). The recovery of poly-P in sediment samples to which *Anabaena* cultures had been added showed some deviation from a 1:1 expected:observed ratio, particularly when 2 ml of the culture were added (ratio of 0.79). However, the recovery improved to 1.12:1 when a larger volume was added (5 ml), probably owing to the inhomogeneous nature of the cyanobacteria culture. In contrast, the addition of 500 mg of trimetaphosphate to the control sediment did not significantly increase ( $p > 0.05$ ) the percentage found for the observed poly-P fraction. Consequently, biologically produced poly-P can be associated with the fraction of poly-P extracted through use of this procedure.

**Key words:** Polyphosphate, EDTA method, P-fractionation, sediment.

### RESUMEN

#### Análisis secuencial para cuantificar el polifosfato biológico en muestras de sedimento

El polifosfato (poly-P) es un compuesto de reserva de fosfato ampliamente distribuido entre células procariotas y eucariotas. Sin embargo, aún no está bien definida la metodología para su cuantificación en muestras de sedimento, debido a la dificultad de encontrar un único extractante para compuestos específicos de P que no altere a los demás. En el presente trabajo se sigue un procedimiento analítico para medir poly-P en cultivos de algas que posteriormente fue modificado para estudiar el poly-P en los sedimentos mediante el método EDTA de fraccionamiento secuencial de P y que permite extraer los compuestos de P inorgánico (P adsorbidos a Fe y Ca) antes de cuantificar el poly-P. Hemos utilizado esta metodología en suspensiones que contenían poly-P producido biológicamente, como cultivos de laboratorio enriquecidos en P de *Synechocystis* PCC 6803 (Cianobacteria), *Anabaena variabilis* (Cianobacteria), y *Chlorella* sp. (Clorofita), así como en fangos activados de depuradora. Siguiendo este procedimiento, las concentraciones de poly-P en los cultivos de *Synechocystis* PCC 6803, *Anabaena*

variabilis, y *Chlorella sp.* supusieron el 15.4, 6.2 y 7.2 % de la suma de todas las fracciones de P, respectivamente, mientras que los fangos activados alcanzaron porcentajes menores (3.7%). Se enriquecieron suspensiones de sedimento natural con volúmenes conocidos de las anteriores suspensiones y con un poly-P estándar comercialmente disponible (Trimetafosfato); se comparó, en muestras duplicadas, la composición de P de estas suspensiones con sus correspondientes muestras de sedimento control. La concentración de poly-P determinada en las muestras de sedimento control fue extremadamente baja ( $0.2 \mu\text{g g}^{-1}$  p.s.), pero ésta aumentaba en todas las suspensiones enriquecidas con las muestras biológicas, en particular con *Synechocystis* (más de  $220 \mu\text{g g}^{-1}$  p.s.). La ratio entre las concentraciones de poly-P esperadas y observadas en las muestras enriquecidas no fue exactamente 1:1, sino 0.79 al añadir 2 ml y 1.12 al incrementar el volumen añadido de cultivo de *Anabaena* (5 ml) debido, probablemente, a la falta de homogeneidad del cultivo de cianobacterias. Sin embargo, la adición de 500 mg de trimetafosfato, un compuesto sintético, a la muestra control de sedimento no incrementó significativamente ( $p > 0.05$ ) el porcentaje de la fracción de poly-P. Por tanto, el poly-P producido biológicamente se puede asociar a la fracción de poly-P extraída siguiendo el procedimiento aquí presentado.

**Palabras clave:** Polifosfato, método EDTA, fraccionamiento de P, sedimento.

#### Abbreviations used:

Abbreviation	Description
Tot-P	Total P
i-P <sub>diss</sub>	Dissolved inorganic phosphate
org-P	Organic phosphate compounds
FeOOH $\approx$ P	Iron-bound phosphate
CaCO <sub>3</sub> $\approx$ P	Calcium-bound phosphate
org-P $\rightarrow$ acid	Organic phosphate compounds soluble in acid
org-P $\rightarrow$ EDTA	Sum of organic phosphate compounds soluble in EDTA solutions
org-P $\rightarrow$ NaOH	Organic phosphate compounds soluble in hot NaOH
org-P <sub>res</sub>	Residual organic phosphate compounds
Tot-P $\rightarrow$ TCA	Total P in the hot TCA extract
TCA	Trichloroacetic acid

## INTRODUCTION

Polyphosphate (poly-P) is a linear orthophosphate polymer containing a variable number of phosphate residues that are linked by energy-rich phosphoanhydride bonds. This compound is widespread in living organisms, particularly microorganisms, and plays a multifunctional role in phosphate and energy storage, cation sequestration, cell envelope formation, membrane transport, and metabolic regulation (Kornberg 1995). Low- and high-molecular weight poly-P occur in a variety of locations within microbial cells, from cell membrane to nucleus, as do poly-P-utilising enzymes (Kulaev & Kulakovskaya 2000). The function of poly-P as a phosphate reserve in prokaryotic and eukaryotic cells is well known.

It has long been established that the intracellular concentration of poly-P depends strongly on the phosphate concentration of the medium in laboratory cell cultures (Kulaev *et al.* 2004). The extrapolation of this relationship to natural systems remains problematic. Attempts to use the concentration of intracellular phosphate reserve compounds in algae as an indication of ecosystem trophic level and P-availability to phytoplankton have produced contradictory results (Kenney *et al.* 2001, Waters *et al.* 2005, Selig *et al.* 2006). The storage of poly-P in cells is, however, caused by the transfer of cells from conditions of starvation to a phosphate-rich culture medium and N shortage (Kuesel *et al.* 1989). The degree of poly-P storage in algae is not significantly affected by variations in phosphate con-

centration in the medium (Eixler *et al.* 2005). Incorporation of external phosphate into the cell poly-P pool proceeds in various steps: transport through the cell membrane, phosphorylation, and polymerisation or elongation of the poly-P chain (Plaetzer *et al.* 2005). Theoretically, this uptake mechanism enables the organisms to maintain continuous growth, but under natural conditions it is more likely that P uptake and growth are decoupled when growth is limited by the lack of another nutrient, often N.

Nuclear magnetic resonance spectroscopy ( $^{31}\text{P}$ -NMR), developed for the determination of poly-P in cell cultures, has been applied to the detection of this compound in soil and sediment. However, it has long been known that great care must be taken to interpret quantifications of organic phosphate compounds because the extractant used will greatly affect both the concentration and the forms of P obtained in a  $^{31}\text{P}$ -NMR analysis (Cade-Menun & Preston 1996, Golterman 2006). The complexity of the mixture of phosphate compounds in sediment makes it impossible for a single extractant to dissolve all P forms or to extract a specific P compound without alteration of others.  $^{31}\text{P}$ -NMR results for these substances in the sediment are only correct if other interfering compounds can be excluded, e.g., by a preceding purification extraction (Golterman, 2006). Thus, the quantification of organic phosphate compounds in the sediment first requires a sequential procedure to ensure that main inorganic phosphate compounds have been previously extracted (i.e., Fe- and Ca-bound phosphate). This fact notwithstanding, sediment organic phosphate compounds are often quantified by using  $^{31}\text{P}$ -NMR after a direct extraction with a mixture of NaOH with or without EDTA (Ahlgren *et al.* 2006, Reitzel *et al.* 2006, Hupfer *et al.* 2007). It is difficult, if not impossible, to compare results from different studies with NMR in which different extractants were used when identifying and quantifying organic P-compounds in the sediment. The present work follows an analytical procedure developed to measure poly-P in algal cultures (Golterman 1960) and later modified for sediment (Golterman 2006). Several samples of biologically pro-

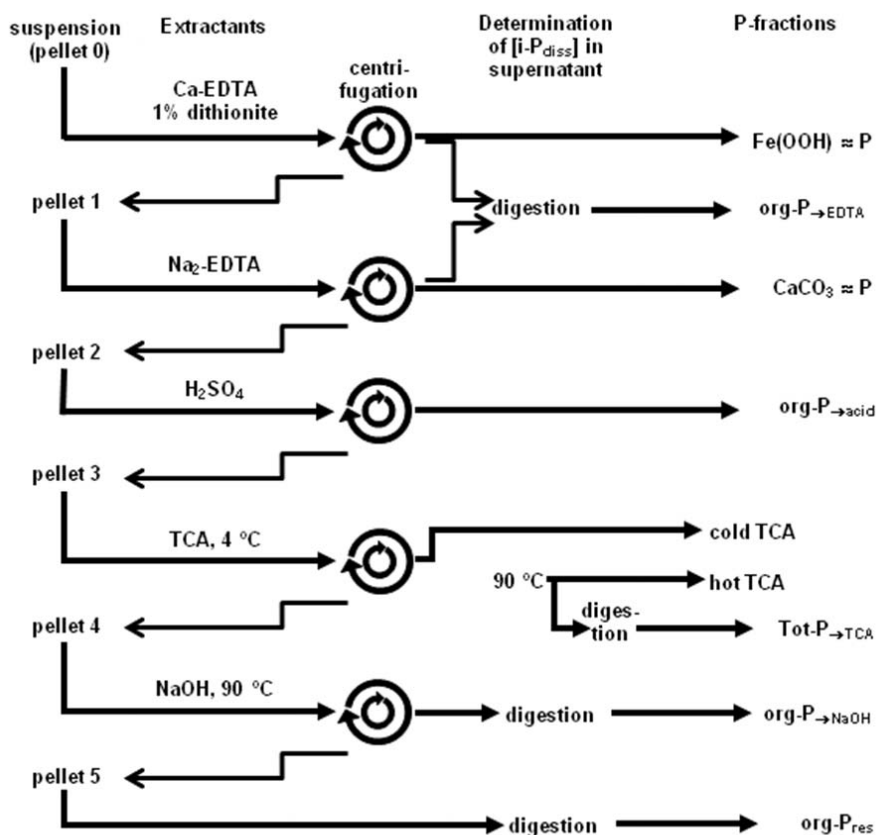
duced poly-P were also analysed to provide positive controls for the extraction of poly-P from the sediment. This biologically produced poly-P came from different sources: activated sludge, a natural phytoplankton bloom, and cell cultures of *Synechocystis* PCC 680, *Anabaena variabilis* and *Chlorella* sp. Additionally, sediment samples were augmented with cell cultures, activated sludge, and a commercially available synthetic poly-P standard compound (Trimetaphosphate) to test for possible interference with the determination of poly-P in the sediment.

## MATERIAL AND METHODS

Two cultures of cyanobacteria (*Synechocystis* strain PCC 6803 and *Anabaena variabilis*) were grown at 30 °C with BG-11c medium (Rippka *et al.* 1979) in 750 ml flasks. The cultures were bubbled with a continuous stream of 1 % (v/v)  $\text{CO}_2$  in air under continuous illumination ( $50 \mu\text{E m}^{-2} \text{s}^{-1}$ ). *Chlorella* sp. was cultured with Sueoka medium in 1000 ml flasks (Sueoka *et al.* 1967). Therefore, these culture media contained a P buffer. Once exponential growth ended, cells were incubated for 12-24 h in light with extra phosphate added to their culture medium to a final concentration of  $5 \text{ mg l}^{-1}$  of P by adding the corresponding volume of a  $\text{K}_2\text{HPO}_4$  solution (1.6 mM). Then, cultures were centrifuged at 12000 rpm for 15 min to recover the cells in pellets that were washed with tap water ( $< 0.1 \mu\text{g l}^{-1}$  of  $\text{i-P}_{\text{diss}}$ ,  $0.3 \text{ mS cm}^{-1}$ ) after each centrifugation. A final cell suspension of about 200 ml was obtained for each culture. In order to remove  $\text{i-P}_{\text{diss}}$  and phosphate associated with the cell surface, 25 ml of trichloroacetic acid (TCA 5 %) was added to 10 ml of each cell suspension in several replicates and kept at 3-4 °C for 0.5-2 h. Then, cell suspensions were centrifuged at 12000 rpm for 15 min, and supernatants were collected in a 200 ml flask for determination of  $\text{i-P}_{\text{diss}}$  concentrations. Each pellet was resuspended in 25 ml of 70 % ethanol at 90 °C for 10-20 min to inactivate enzyme activity and to remove lipid-associated P (Golterman 1960) and later centrifuged (12000 rpm, 15 min). Supernatants were

collected in a 200 ml flask for determination of  $i\text{-P}_{\text{diss}}$  concentrations. Pellets were resuspended in distilled water to obtain the final cell suspensions. The dry weight of these suspensions was determined gravimetrically in 3-6 replicates of 1 ml-aliquot each. Tot-P concentration was measured in 2-6 replicates of 1 ml-aliquot each as  $i\text{-P}_{\text{diss}}$  following the method of Murphy & Riley (1962) after acid digestion with 0.5 M  $\text{H}_2\text{SO}_4$  and  $\text{K}_2\text{S}_2\text{O}_8$  (0.5-1 g) at 120 °C for 4 h (De Groot & Golterman 1990). The optical density at 882 nm was read in a HITACHI U-2000 Spectrophotometer using either a 1 cm or 10 cm cuvette. The P-fractionation of the final cell suspension was performed according to the EDTA method for sequential extraction of P in the sediment (Golterman 1996) and later modified to measure poly-P

(Golterman 2006). The results are the average of a duplicate of 10 ml aliquots for *Chlorella* sp., 2 ml aliquots for *Synechocystis* PCC 6803 and 5 ml aliquots for *Anabaena*. In the first extraction step, 25 ml of a solution of 0.05 M Ca-EDTA (pH 7.8) containing 1 % dithionite was added to each aliquot (pellet 0, Fig. 1). This extraction was performed during 1-2 h over continuous shaking in a rotor. The suspensions were then centrifuged (12000 rpm, 15 min) and the concentration of  $i\text{-P}_{\text{diss}}$  was measured in each supernatant in order to determine the  $\text{Fe}(\text{OOH}) \approx \text{P}$ . Next, 25 ml of 0.1 M  $\text{Na}_2\text{-EDTA}$  (pH 5) was added to the previous pellet and shaken continuously during 2-6 h. The suspensions were centrifuged again. The measured concentration of  $i\text{-P}_{\text{diss}}$  in the supernatant corresponds to the fraction of  $\text{CaCO}_3 \approx \text{P}$ .



**Figure 1.** The EDTA method for sequential P-fractionation of sediment (Golterman 1996) with some modifications for the quantification of poly-P as proposed by Golterman (2006). *Método EDTA para la extracción secuencial de las fracciones P sedimentario (Golterman 1996) con algunas modificaciones para la cuantificación de poly-P propuestas por Golterman (2006).*

The concentration of Tot-P was also measured in the supernatants extracted with EDTA in order to calculate the organic P-fraction dissolved by the reagent itself ( $\text{org-P}_{\rightarrow \text{EDTA}}$ ) as the difference between the Tot-P and  $\text{i-P}_{\text{diss}}$  concentrations. Next, 25 ml of 0.5 M  $\text{H}_2\text{SO}_4$  was added to the previous pellet (1 h, continuous shaking). The suspensions were centrifuged as usual, and the concentration of  $\text{i-P}_{\text{diss}}$  in the supernatant was measured to obtain the organic P-fraction soluble in  $\text{H}_2\text{SO}_4$  ( $\text{org-P}_{\rightarrow \text{acid}}$ ) and to ensure that the extraction of the previous P-fractions was completed. Then, 25 ml of TCA (5 %) were added to the previous pellet, kept at 3–4 °C for 45 min, centrifuged as usual and the concentration of  $\text{i-P}_{\text{diss}}$  measured in the supernatant (cold TCA extraction). The same suspension was then heated in a water bath at 90 °C for another 45 min. The concentration of  $\text{i-P}_{\text{diss}}$  in the supernatant was measured after centrifugation (hot TCA extraction). The difference between the  $\text{i-P}_{\text{diss}}$  concentration in the hot and cold TCA extractions equals the poly-P fraction, according to Golterman (1960), who followed the acid-soluble specific method of MacFarlane (1936 in Kulaev *et al.*, 2004). The concentration of Tot-P in the supernatant with hot TCA was determined after digestion ( $\text{Tot-P}_{\rightarrow \text{TCA}}$ ). Next, 25 ml of 2 M NaOH was added to the previous pellet and kept at 90 °C for 2 h. The suspensions were centrifuged, and each supernatant was brought to a final volume of 50 ml with  $\text{H}_2\text{O}$ . The concentration of Tot-P after digestion of the supernatant yielded the organic P-fraction soluble in hot NaOH ( $\text{org-P}_{\rightarrow \text{NaOH}}$ ). The next pellet was digested with 0.5 M  $\text{H}_2\text{SO}_4$  and 1 g  $\text{K}_2\text{S}_2\text{O}_8$  at 120 °C for the determination of Tot-P. This fraction was termed the residual organic P-fraction ( $\text{org-P}_{\text{res}}$ ). Owing to the high concentrations of  $\text{i-P}_{\text{diss}}$  found in the  $\text{Fe}(\text{OOH})\approx\text{P}$  and in the  $\text{org-P}_{\rightarrow \text{NaOH}}$ , both steps were repeated several times to ensure a complete extraction. The difference between the sum of P-fractions and the Tot-P concentration of these cell cultures was lower than 15 %.

A water sample of 1.5 L was collected from the shore of Dulce pond (Doñana National Park, SW Spain) during a phytoplankton bloom in September 2007. An aliquot was preserved with

lugol *in situ* for taxonomic determination with an optical microscope (1000× of magnification). *Aphanocapsa* aff. *holsatica* (Cyanobacteria) was the dominant taxon in this bloom. A cell preparation was obtained following the same procedure as that applied to the laboratory cultures (centrifugation, 5 % TCA and ethanol pretreatments), except that no P incubation was performed. Then, dry weight, P-fraction and Tot-P were determined as mentioned above in duplicate with 1 ml aliquots (Fig. 1). The difference between the sum of P-fractions and the Tot-P concentration of the phytoplankton suspension was always lower than 10 % and often lower than 5 %. Dry-weight and Tot-P were also determined for the original water sample before obtaining the cell suspension.

A sample of activated sludge was provided by the laboratory staff of the municipal sewage-treatment plant (E.D.A.R. Tablada, Sevilla) and analysed a few hours after collection. A suspension of this activated sludge was obtained and pretreated prior to P-fractionation (in duplicate with 5 ml aliquots) as mentioned above (Fig. 1). Again, the difference between the sum of P-fractions and the Tot-P concentration of the activated sludge was less than 10 %.

A 200 ml sediment suspension was made with  $\text{H}_2\text{O}$  and dry sediment collected from a temporary pond (Dulce), once the pond had naturally dried up, to represent the control sediment samples (SED). A volume of 10 ml of the final cell suspension of *Synechocystis* PCC 6803 was added to 10 ml of sediment suspension in duplicate (SED+SYN). Duplicate sediment samples were also augmented with 5 ml of the final cell suspension of *Chlorella* (SED+CHL), 1.5 ml of the activated sludge (SED+ACT), and Na-trimetaphosphate (SED+TMP) in increasing quantities (100, 250, and 500 mg). The dry weight of each final suspension was evaluated gravimetrically (3 replicates of 1 ml each). A more careful recovery test was also made with sediment samples augmented using 2 different volumes of *Anabaena* cell culture: 2 and 5 ml of cell culture, with an average dry weight of  $0.0101 \text{ g ml}^{-1}$ , were added, in duplicate, to a known amount of control sediment (2.0135 g on

**Table 1.** Average concentration of each P-fraction ( $\mu\text{g g}^{-1}$  d.w.) in laboratory cell cultures (*Synechocystis* PCC 6803, *Anabaena variabilis*, *Chlorella* sp.), a natural phytoplankton bloom, and activated sludge. *Concentración media de cada fracción ( $\mu\text{g g}^{-1}$  p.s.) en los cultivos celulares de laboratorio (*Synechocystis* PCC 6803, *Anabaena variabilis*, *Chlorella* sp.), en un bloom de fitoplancton y en fangos activados.*

P-fraction	<i>Synechocystis</i> PCC 6803	<i>Anabaena</i> <i>variabilis</i>	<i>Chlorella</i> sp.	Phytoplankton	Activated sludge
FeOOH $\approx$ P	2539	1677	1750	46	757
CaCO <sub>3</sub> $\approx$ P	683	37	51	63	46
org-P $\rightarrow$ EDTA	12836	3249	2211	761	46602
org-P $\rightarrow$ acid	135	26	6	2	25
Tot-P $\rightarrow$ TCA	4012	9651	6385	2108	9490
org-P $\rightarrow$ NaOH	1304	2042	1295	1419	1815
org-P <sub>res</sub>	56	222	277	57	69
$\Sigma$ P-fractions	21566	16904	11975	4456	58805

average). The final dry weight of each augmented suspension was the sum of the dry weight in each control sediment sample plus the dry weight that corresponded to the added volume of the cell culture. P-fractionation was carried out at the same time for each amended sample and the cell culture. The concentration of Tot-P was carried out as described above using 2 replicates of 1 ml for each suspension. On average, the difference between the sum of P-fractions and the Tot-P concentration of the amended sediment suspensions was less than 11 %.

## RESULTS

The concentration of Tot-P was highest in the sample of activated sludge, had intermediate values for the cell cultures of *Synechocystis* sp. PCC 6803, *Anabaena variabilis* and *Chlorella* sp. growing in a P-enriched culture medium, and was lowest in the natural phytoplankton bloom (Table 1). As expected, the sum of organic P-fractions constituted the largest portion in these samples, ranging from 78 % in the *Chlorella* culture to 99 % of the sum of all P-fractions in the activated sludge, but each suspension had a different proportion of organic P-fractions (Table 1). The activated sludge showed the highest percentage of org-P $\rightarrow$ EDTA, whereas the cultures of *Chlorella* and *Anabaena* and the phytoplankton bloom were richer in Tot-P $\rightarrow$ TCA.

Once main inorganic P-fractions (FeOOH  $\approx$  P and CaCO<sub>3</sub>  $\approx$  P) and organic phosphate compounds soluble in acid (org-P $\rightarrow$ acid) had been extracted, the concentration of poly-P was determined as the difference between a cold and a hot TCA extraction (Table 2). The percentage of poly-P was the highest for the culture of *Synechocystis* (15.4 %) followed by the culture of *Chlorella* (7.2 %) and *Anabaena* (6.2 %), whereas the phytoplankton bloom and the activated sludge showed lower proportions (3.8 and 3.7 %, respectively). This P-fractionation procedure was performed on sediment alone (as a control) and on sediment suspensions augmented with biological samples (cell cultures and activated sludge) and with a standard syn-

**Table 2.** Average concentration of i-P<sub>diss</sub> ( $\mu\text{g g}^{-1}$  d.w.) extracted with TCA, a procedure that yielded measurements of the poly-P pool from several biological sources: three laboratory cell cultures (*Synechocystis* PCC 6803, *Anabaena variabilis*, *Chlorella* sp.), a natural phytoplankton bloom, and activated sludge. *Concentración media de i-P<sub>diss</sub> ( $\mu\text{g g}^{-1}$  p.s.) extraída con TCA y su correspondiente fracción de poly-P en distintos materiales biológicos: tres cultivos celulares (*Synechocystis* PCC 6803, *Anabaena variabilis*, *Chlorella* sp.), un bloom de fitoplancton y en fangos activados.*

	cold TCA	hot TCA	poly-P
<i>Synechocystis</i> PCC 6803	48	3375	3327
<i>Anabaena variabilis</i>	26	1067	1041
<i>Chlorella</i> sp.	76	936	860
Phytoplankton	25	197	172
Activated sludge	132	2331	2199

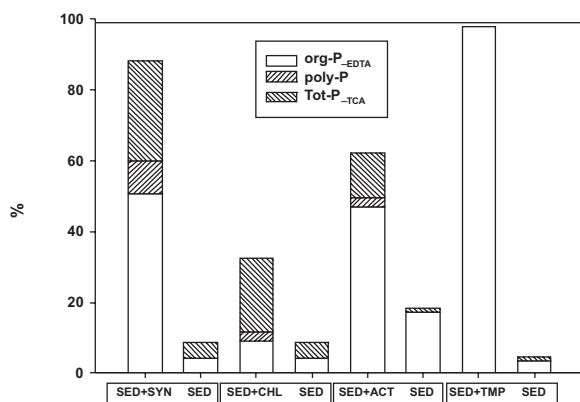
**Table 3.** Average concentration of P-fractions ( $\mu\text{g g}^{-1}$  d.w.) in sediment augmented with *Synechocystis* PCC 6803 (SED+SYN), *Anabaena variabilis* (SED + ANA), *Chlorella* sp. (SED+CHL), activated sludge (SED+ACT), and trimetaphosphate (SED+TMP) and their corresponding control with sediment alone (SED). *Concentración media de las fracciones de P ( $\mu\text{g g}^{-1}$  p.s) en el sedimento enriquecido con *Synechocystis* PCC 6803 (SED+SYN), *Anabaena variabilis* (SED + ANA), *Chlorella* sp. (SED+CHL), fango activado (SED+ACT), y trimetafosfato (SED+TMP), así como sus correspondientes sedimentos control (SED).*

	SED	SED+SYN	SED	SED+ANA	SED	SED+CHL	SED	SED+ACT	SED	SED+TMP
$\text{FeOOH}\approx\text{P} + \text{CaCO}_3 \approx\text{P}$	89	220	28	71	89	154	28	40	61	409
$\text{org-P}_{\rightarrow\text{EDTA}}$	10	1164	14	72	10	33	14	161	6	24608
$\text{org-P}_{\rightarrow\text{acid}}$	9	9	1	3	9	4	1	4	4	5
$\text{Tot-P}_{\rightarrow\text{TCA}}$	10	648	1	217	10	76	1	44	2	6
$\text{org-P}_{\rightarrow\text{NaOH}} + \text{org-P}_{\text{res}}$	114	261	37	123	114	104	37	94	95	110

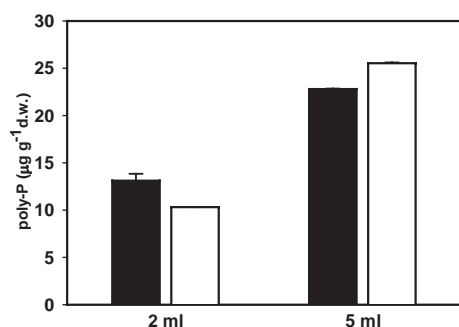
thetic poly-P compound (Trimetaphosphate) to test for interference with the determination of biologically-produced poly-P in the sediment (Table 3). The largest P-fraction was the sum of  $\text{org-P}_{\rightarrow\text{NaOH}}$  and  $\text{org-P}_{\text{res}}$  in all control sediment samples. The augmentation of the control sediment suspensions changed the P-fraction composition (Table 3), and this change was particularly large for the percentages of  $\text{org-P}_{\rightarrow\text{EDTA}}$  and  $\text{Tot-P}_{\rightarrow\text{TCA}}$  (Fig. 2). As expected for the dry sediment of a temporary pond, the poly-P concentration was extremely low in all control sedi-

ment samples, almost approaching the detection limit ( $0.2 \mu\text{g g}^{-1}$  d.w.), but its concentration increased in the sediment suspensions augmented with biological samples. In contrast, the sediment augmented with the synthetic trimetaphosphate showed a highly significant increase in the  $\text{org-P}_{\rightarrow\text{EDTA}}$  fraction, whereas the percentage of poly-P and  $\text{Tot-P}_{\rightarrow\text{TCA}}$  did not increase significantly ( $p > 0.05$ ) compared to the control. Further additions of trimetaphosphate (up to 500 mg) to the control sediment suspension did not yield a significant increase ( $p > 0.05$ ) in the proportion of poly-P and  $\text{Tot-P}_{\rightarrow\text{TCA}}$ , whereas the  $\text{org-P}_{\rightarrow\text{EDTA}}$  fraction accounted for nearly the sum of all P-fractions (97.8-98.3 %).

The recovery of poly-P in sediment samples augmented with the *Anabaena* cell culture did not show an exact 1:1 ratio between the expected and observed values. This ratio was 0.79 and 1.12 for the addition of 2 and 5 ml of the culture, respectively. The addition of 2 ml of the cell culture implied an expected poly-P concentration of only  $10 \mu\text{g g}^{-1}$  d.w., probably too low a concentration to be detected without error, whereas the larger volume of 5 ml yielded a more precise and accurate result (Fig. 3). In contrast, the addition of up to 500 mg of Na-trimetaphosphate, a synthetic standard cyclic P-compound, did not produce a significant increase in the observed poly-P concentration because the added P appeared in a different P-fraction ( $\text{org-P}_{\rightarrow\text{EDTA}}$ ) at the beginning of the P-fractionation. Consequently, biologically produced poly-P can be associated with the fraction of poly-P extracted through use of this procedure.



**Figure 2.** Percentage of target P-fractions in each test of sediment augmented with *Synechocystis* PCC 6803 (SED+SYN), *Chlorella* sp. (SED+CHL), activated sludge (SED+ACT), and trimetaphosphate (SED+TMP) and their corresponding control with sediment alone (SED). *Porcentaje de las fracciones de P en cada cultivo de sedimento enriquecido con *Synechocystis* PCC 6803 (SED+SYN), *Chlorella* sp. (SED+CHL), fango activado (SED+ACT), y trimetafosfato (SED+TMP), así como sus correspondientes sedimentos control (SED).*



**Figure 3.** Recovery test of poly-P in sediment samples amended with 2 and 5 ml of *Anabaena* cultures. Mean concentration and SE of the detected poly-P after the augmentation (black fill) and the calculated mean and SE of the expected concentration (white fill) for each added volume. *Recuperación de poly-P en muestras de sedimento enriquecidas con 2 y 5 ml del cultivo de Anabaena: se muestra la concentración media y desviación estándar (SE) del poly-P detectado (en negro) y la media y desviación estándar (SE) de la concentración esperada (en blanco) según el correspondiente volumen añadido.*

## DISCUSSION

This study found the highest percentage of stored poly-P in the *Synechocystis* culture (15.4 %). Storage of poly-P in laboratory cultures of autotrophic organisms varies widely according to the literature. The proportion of poly-P has been reported to be higher at faster growth rates (Rhee 1973 in Thompson *et al.* 1994). In some cyanobacterial cultures, poly-P represented 60 % of the Tot-P in cells of *Oscillatoria redekei*, 10 % in *Syneococcus*, and between 3 and 6 % in *Anabaena flos-aquae* (Thompson *et al.* 1994). In *Scenedesmus* cells, between 5 and 32 % of cellular-P accounted for poly-P (Golterman 1960). This variability results from multiple factors, including the extent of P limitation in the cell cultures, growth rate, N starvation, and specific physiological adaptations (Kuesel *et al.* 1989, Kulaev *et al.* 2004). Comparison with other results is difficult because poly-P is quantified by a diversity of methods in the literature: *in vivo* NMR, NMR after a variety of extractants (NaOH, EDTA, TCA), hot water, cold/hot TCA treatment (Hupfer *et al.* 2008). The elegance of the method presented here lies in the fact that the same reagent was used for the prepurification of the pellet (TCA at 4 °C) and the extraction of poly-P (TCA at 90 °C) from the same su-

pernatant. Only temperature was changed to allow for the extraction of this compound, as poly-P is hydrolysed  $10^5$ - $10^6$  times faster at 100 °C than at 0 °C (Greenwood & Earnshaw 1984). After hydrolysis, the TCA extract probably yielded a mixture of compounds (defined here as Tot-P $\rightarrow$ TCA) with a higher proportion of org-P compounds in the *Chlorella* culture than the *Synechocystis* culture. Nucleotide-P and sugar-P are reported to be extracted together with poly-P in TCA-soluble fractions from cell cultures of photoautotrophs such as *Scenedesmus quadricauda* (Golterman 1960) and the cyanobacterium *Anabaena flos-aquae* (Thompson *et al.* 1994).

In the sequential procedure presented here, the main inorganic P-fractions had been previously extracted by two sequential treatments with chelating agents (Ca-EDTA and Na<sub>2</sub>-EDTA) followed by an extraction with diluted H<sub>2</sub>SO<sub>4</sub> (org-P $\rightarrow$ acid) to prevent contamination of subsequent steps with traces of previous fractions. As expected, this diluted-acid washing accounted for a very small percentage of all P-fractions (0.1-0.6 %), similar to that of the final residual pellet (org-P<sub>res</sub>). The use of EDTA allowed for the quantification of a P-fraction soluble in EDTA but not detectable until hydrolysed, and thus defined here as an org-P fraction (org-P $\rightarrow$ EDTA). However, in biological samples, it may correspond to the inorganic phosphate pool of cells (Lawrence *et al.*, 1998). *In vivo* <sup>31</sup>P-NMR spectroscopy of *Synechocystis* sp. strain PCC 6308 has indicated that inorganic phosphate, but not poly-P with a molecular weight > 1000 Da, leaks from the cell as the result of treatment with EDTA (Lawrence *et al.* 1998). The reason that this cellular inorganic phosphate requires a hydrolysis for its detection is unclear. The low-molecular-weight trimetaphosphate also required hydrolysis for its detection. Trimetaphosphate is a synthetic cyclic poly-P compound and appears to be more stable in aqueous solution than linear polyphosphates. The assignment of peaks for poly-P in <sup>31</sup>P-NMR spectroscopy of soil and sediment extractions has been traditionally based on a standard of commercial trimetaphosphate (Adams & Byrne, 1989; Cade-Menun & Preston, 1996). In our study, however, we used sev-



eral standards (a commercial trimetaphosphate, cell cultures grown in a P-rich medium and activated sludge) to allow an analytical comparison for biologically stored poly-P. These biological suspensions provided a positive control in order to test the efficiency of this procedure for the detection of cell poly-P. The trimetaphosphate provided a negative control, as it was not produced biologically. Although this procedure does not determine poly-P molecular size or separate poly-P from different intracellular locations, it can be a useful tool to assess the relevance of biologically produced poly-P. This poly-P of biological origin is eventually deposited in the sediment under natural conditions.

The deviation from a 1:1 ratio between the expected and detected poly-P concentrations in sediment samples augmented with *Anabaena* was slightly higher when 2 ml rather than 5 ml of cell culture were added. The former addition implied a lower targeted poly-P concentration (10 and 26  $\mu\text{g g}^{-1}$  d.w., respectively) and was thus more likely to accumulate errors. Nevertheless, only 1  $\mu\text{g g}^{-1}$  d.w. of difference was found to occur between the replicates for the poly-P concentration detected in the duplicated suspension amended with 2 ml, whereas no such error in precision occurred with the 5 ml addition. The calculation of recovery ratios for augmented preparations made using other P sources was hampered by several factors: *a*) the low biomass of the biological sources implied very low targeted poly-P concentrations; *b*) the cell cultures produced inhomogeneous suspensions that caused low precision among replicates; *c*) the number of replicates was severely limited by the low biomass of the cell cultures. The recovery test was conducted for the augmentations involving *Anabaena* because these cultures produced the least inhomogeneous suspensions. Indeed, dry weight was calculated with a precision 5–10 times higher than the precision obtained with the other P sources.

## CONCLUSIONS

The application of this methodology allows the quantification of the sediment P-fraction that cor-

responds to the P stored by aquatic microorganisms. The method is feasible for this purpose because it has been tested with biologically produced poly-P (positive control) and a synthetic trimetaphosphate (negative control). The advantage of this method is that it follows the EDTA method of sediment P-fractionation long used in natural aquatic environments. Moreover, it ensures that the main inorganic P-fractions (i.e., Fe- and Ca-bound phosphate) have been extracted prior to the determination of org-P fractions. Additionally, the quantification of biological poly-P is carried out sequentially in the same supernatant after a change in temperature combined with extraction in cold TCA as a specific blank for each sample. Therefore, this procedure is suitable for ecological studies that aim at the quantification of biologically produced poly-P in aquatic systems.

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